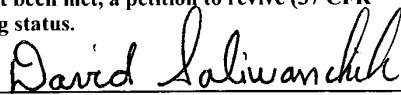


JC07 Rec'd PCT/PTO 08 MAR 2002

FORM PTO-1390 (REV. 9-2001)		U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE		ATTORNEY'S DOCKET NUMBER GJE-88	
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371				U.S. APPLICATION NO. (If known, see 37 CFR 1.5) 10/070568	
INTERNATIONAL APPLICATION NO. PCT/GB00/03460		INTERNATIONAL FILING DATE 8 September 2000		PRIORITY DATE CLAIMED 8 September 1999	
TITLE OF INVENTION MONOMERIC ANALOGUES OF HUMAN INSULIN					
APPLICANT(S) FOR DO/EO/US You-Min Feng and You-Shang Zhang					
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:					
<p>1. <input checked="" type="checkbox"/> This is a FIRST submission of items concerning a filing under 35 U.S.C. 371.</p> <p>2. <input type="checkbox"/> This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371.</p> <p>3. <input type="checkbox"/> This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below.</p> <p>4. <input type="checkbox"/> The US has been elected by the expiration of 19 months from the priority date (Article 31).</p> <p>5. <input checked="" type="checkbox"/> A copy of the International Application as filed (35 U.S.C. 371(c)(2))</p> <p style="margin-left: 20px;">a. <input type="checkbox"/> is attached hereto (required only if not communicated by the International Bureau).</p> <p style="margin-left: 20px;">b. <input checked="" type="checkbox"/> has been communicated by the International Bureau.</p> <p style="margin-left: 20px;">c. <input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US).</p> <p>6. <input type="checkbox"/> An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).</p> <p style="margin-left: 20px;">a. <input type="checkbox"/> is attached hereto.</p> <p style="margin-left: 20px;">b. <input type="checkbox"/> has been previously submitted under 35 U.S.C. 154(d)(4).</p> <p>7. <input type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))</p> <p style="margin-left: 20px;">a. <input type="checkbox"/> are attached hereto (required only if not communicated by the International Bureau).</p> <p style="margin-left: 20px;">b. <input type="checkbox"/> have been communicated by the International Bureau.</p> <p style="margin-left: 20px;">c. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired.</p> <p style="margin-left: 20px;">d. <input type="checkbox"/> have not been made and will not be made.</p> <p>8. <input type="checkbox"/> An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371 (c)(3)).</p> <p>9. <input checked="" type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)) _____</p> <p>10. <input type="checkbox"/> An English language translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).</p> <p>Items 11 to 20 below concern document(s) or information included:</p> <p>11. <input type="checkbox"/> An Information Disclosure Statement under 37 CFR 1.97 and 1.98.</p> <p>12. <input type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.</p> <p>13. <input checked="" type="checkbox"/> A FIRST preliminary amendment.</p> <p>14. <input type="checkbox"/> A SECOND or SUBSEQUENT preliminary amendment.</p> <p>15. <input type="checkbox"/> A substitute specification.</p> <p>16. <input type="checkbox"/> A change of power of attorney and/or address letter.</p> <p>17. <input type="checkbox"/> A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 37 CFR 1.821 - 1.825.</p> <p>18. <input type="checkbox"/> A second copy of the published international application under 35 U.S.C. 154(d)(4).</p> <p>19. <input type="checkbox"/> A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).</p> <p>20. <input checked="" type="checkbox"/> Other items or information: Certificate of Mailing by Express Mail</p>					

U.S. APPLICATION NO. 10/070568		INTERNATIONAL APPLICATION NO. PCT/GB00/03460		ATTORNEY'S DOCKET NUMBER GJE-88	
21. <input checked="" type="checkbox"/> The following fees are submitted: BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)): Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO. \$1040.00 International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO \$890.00 International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO \$740.00 International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) \$710.00 International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) \$100.00 ENTER APPROPRIATE BASIC FEE AMOUNT =				CALCULATIONS PTO USE ONLY <div style="border: 1px solid black; padding: 5px; width: fit-content; margin: 0 auto;">\$890.00</div>	
Surcharge of \$130.00 for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(e)).					
CLAIMS	NUMBER FILED	- NUMBER EXTRA	RATE	\$	
Total claims	17 - 20 =	0	x \$18.00	\$0.00	
Independent claims	3 - 3 =	0	x \$84.00	\$0.00	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ \$270.00	\$0.00	
TOTAL OF ABOVE CALCULATIONS =				\$890.00	
<input type="checkbox"/> Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2.				+	
SUBTOTAL =				\$890.00	
Processing fee of \$130.00 for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(f)).					
TOTAL NATIONAL FEE =				\$890.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property +					
TOTAL FEES ENCLOSED =				\$890.00	
				Amount to be refunded:	\$
				charged:	\$
a. <input type="checkbox"/> A check in the amount of \$ _____ to cover the above fees is enclosed. b. <input checked="" type="checkbox"/> Please charge my Deposit Account No. <u>19-0065</u> in the amount of \$ <u>890.00</u> to cover the above fees. A duplicate copy of this sheet is enclosed. c. <input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. <u>19-0065</u> . A duplicate copy of this sheet is enclosed. d. <input type="checkbox"/> Fees are to be charged to a credit card. WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.					
NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137 (a) or (b)) must be filed and granted to restore the application to pending status.					
CORRESPONDENCE ADDRESS:			 SIGNATURE		
CUSTOMER NUMBER 23,557			March 8, 2002	David R. Saliwanchik	
			DATE	NAME	
				31,794	
				REGISTRATION NUMBER	

10/070568-0002
Rec'd PCT/PTO 06 DEC 2002

I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail in an envelope addressed to: U.S. Patent and Trademark Office, Box Sequence, PO Box 2327 Arlington, VA 22202 on December 2, 2002.

David R. Saliwanchik

David R. Saliwanchik, Patent Attorney

SUBMISSION OF SEQUENCE LISTING
UNDER 37 CFR §§1.821-1.825
Patent Application
Docket No. GJE-88
Serial No. 10/070,568

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants : You-Min Feng, You-Shang Zhang
Serial No. : 10/070,568
Filed : March 8, 2002
Conf. No. : 7193
For : Monomeric Analogues of Human Insulin

Box SEQUENCE
Assistant Commissioner for Patents
PO Box 2327
Arlington, VA 22202

SUBMISSION OF SEQUENCE LISTING UNDER 37 CFR §§1.821-1.825

Sir:

Transmitted herewith is a replacement Sequence Listing Under 37 CFR §§1.821 through 1.825 for the above-identified patent application. A Notification of Defective Response Under 35 U.S.C. 371 in the United States Designated/Elected Office (DO/EO/US) was received from the Patent and Trademark Office, and a copy of the Notification is enclosed herewith.

The Sequence Listing is submitted in computer readable format and on paper. I hereby certify that the paper and computer readable copies contain the same information and that no new material is added by this submission.

The Commissioner is hereby authorized to charge any fees under 37 CFR 1.16 or 1.17 as required by this paper to Deposit Account No. 19-0065.

Respectfully submitted,



David R. Saliwanchik
Patent Attorney
Registration No. 31,794
Phone No.: 352-375-8100
Fax No.: 352-372-5800
Address: 2421 N.W. 41st Street, Suite A-1
Gainesville, FL 32606-6669

DRS/la

Attachments: Sequence listing on paper and computer readable format containing the same information; Amendment Under 37 CFR §1.825(a) through (c); copy of Notification of Defective Response.

10/070568
Rec'd PCT/PTO 06 DEC 2002

I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail in an envelope addressed to:

Assistant Commissioner for Patents

Washington, D.C. 20231 on December 2, 2002

David Saliwanchik

David R. Saliwanchik, Patent Attorney

AMENDMENT UNDER 37 CFR

§1.825(a) THROUGH (c)

Patent Application

Docket No. GJE-88

Serial No. 10/070,568

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants : You-Min Feng, You-Shang Zhang
Serial No. : 10/070,568
Filed : March 8, 2002
Conf. No. : 7193
For : Monomeric Analogues of Human Insulin

Box PCT/Box SEQUENCE

Assistant Commissioner for Patents

P.O. Box 2327

Arlington, VA 22202

AMENDMENT UNDER 37 CFR §1.825(a) THROUGH (c)

Sir:

In response to the Notification of Defective Response Under 35 U.S.C. 371 in the United States Designated/Elected Office (DO/EO/US) received in the above-identified patent application, please amend the subject application as follows, in order to comply with the requirements of 37 CFR §§1.821-1.825:

In the Specification

Please substitute the paragraph on page 3, lines 4-13 with the following:

In order to obtain recombinant forms of human insulin analogues according to this invention, target genes were produced. This was accomplished by the "gap double-stranded DNA" method

described by Li Yiping *et al.* (1987, *Biotech. J.* 3:90) which permits site-directed mutations in the HI target gene. Primers specifically designed to give B12Thr, B16Ala and B26Ala were as follows;
For B12Thr (NHI-2): refer to Wang *et al.*, *supra*
For B16Ala (NHI-3): 5' TGA GGC TTT GNN STT GGT TTG CG 3' (SEQ ID No.1) in which N can be any nucleotide (G,A,T or C), and S is C or G.
For B26Ala (NHI-4): 5' GAA AGA GGTT TTC NNS ACT CCT AGG GC 3' (SEQ ID No.2) in which N and S are as defined above.

In the Sequence

Please replace original pages 1-3 (Sequence Listing) with new pages 1-2 attached hereto.

Marked-up Version of Substitute Specification

Please substitute the paragraph on page 3, lines 4-13 with the following:

In order to obtain recombinant forms of human insulin analogues according to this invention, target genes were produced. This was accomplished by the "gap double-stranded DNA" method described by Li Yiping *et al.* [6] (1987, *Biotech. J.* 3:90) which permits site-directed mutations in the HI target gene. Primers specifically designed to give B12Thr, B16Ala and B26Ala were as follows;
For B12Thr (NHI-2): refer to Wang *et al.*, *supra*
For B16Ala (NHI-3): 5' TGA GGC TTT GNN ¥TF STT GGT TTG CG 3' (SEQ ID No.1) in which N can be any nucleotide (G,A,T or C), and ¥ S is C or G.
For B26Ala (NHI-4): 5' GAA AGA GGTT TTC NNY NNS ACT CCT AGG GC 3' (SEQ ID No.2) in which N and ¥ S are as defined above.

10070510/070568
Rec'd PCT/PTO 15 JUL 2002

I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail in an envelope addressed to:

Assistant Commissioner for Patents

Washington, D.C. 20231 on July 8, 2002

David Saliwanchik
David R. Saliwanchik, Patent Attorney

AMENDMENT UNDER 37 CFR

§1.825(a) THROUGH (c)

Patent Application

Docket No. GJE-88

Serial No. 10/070,568

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants : You-Min Feng, You-Shang Zhang
Serial No. : 10/070,568
Filed : March 8, 2002
Conf. No. : 7193
For : Monomeric Analogues of Human Insulin

Box PCT

Assistant Commissioner for Patents

Washington, D.C. 20231

AMENDMENT UNDER 37 CFR §1.825(a) THROUGH (c)

Sir:

In response to the Notification of Missing Requirements Under 35 U.S.C. 371 in the United States Designated/Elected Office (DO/EO/US) received in the above-identified patent application, please amend the subject application as follows, in order to comply with the requirements of 37 CFR §§1.821-1.825:

In the Specification

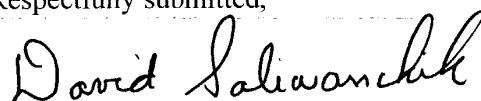
Please replace original pages 1-2 (Sequence Listing) with new pages 1-3 attached hereto.

Remarks

This amendment is made to conform the application with the provisions of 37 CFR §§1.821 through 1.825. I hereby certify that no new material is being added by this submission.

The Commissioner is hereby authorized to charge any fees under 37 CFR 1.16 or 1.17 as required by this paper to Deposit Account No. 19-0065.

Respectfully submitted,



David R. Saliwanchik
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Gainesville, FL 32606-6669

DRS/la

Attachment: New pages 1-3 (Sequence Listing) of the subject specification

10/070568
JC19 Rec'd PCT/PTO 08 MAR 2002

March 8, 2002

Patent Application
Docket No. GJE-88

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants : You-Min Fend and You-Shang Zhang
Docket No. : GJE-88
For : Monomeric Analogues Of Human Insulin

PRELIMINARY AMENDMENT

Please amend the above-identified patent application as follows:

In the Specification

Please add the following paragraph at page 1, above line 2:

This application is a National Stage Application of International Application
Number PCT/GB00/03460, published, pursuant to PCT Article 21(2), in English.

After page 8: Please insert as new page 9 the attached Abstract of the Disclosure.

In the claims

The following amendments are made with respect to the claims in the international application PCT/GB00/03460 attached as Annexes to the International Preliminary Examination Report (IPER). Therefore, please replace existing page 8 of the international application with the amended claim sheet (replacement page 8) of the annex attached to the IPER, and make the following amendments to the pending claims so that they read as follows:

Claim 1 (amended):

An insulin analogue wherein the 16th or 26th amino acid of the B chain of human insulin (Tyr) is substituted by Ala, and which optionally also comprises a deletion at B1(Phe) and/or B30 (Thr).

Claim 2 (amended):

The insulin analogue, according to claim 1, wherein the 26th amino acid of the B chain of human insulin (Tyr) is substituted by Ala.

Claim 3 (amended):

The insulin analogue, according to claim 1, wherein the 26th amino acid is substituted by Ala, and which comprises a deletion at B30.

Claim 4 (amended):

The insulin analogue, according to claim 1, wherein the 16th amino acid of the B chain of human insulin (Tyr) is substituted by Ala.

Claim 5 (amended):

The insulin analogue, according to claim 1, wherein the 16th amino acid is substituted by Ala, and which comprises a deletion at B30.

Please add the following new claims:

6. A method for treating an individual having an insulin deficiency wherein said method comprises administering to the individual an insulin analogue wherein the 16th or 26th amino acid of the B chain of human insulin (Tyr) is substituted by Ala.

7. The method, according to claim 6, wherein said analogue has a deletion at B1 (Phe) or a deletion at B30 (Thr), or a deletion at both B1 and B30.

8. The method, according to claim 6, wherein, at the 26th amino acid, the analogue is substituted by Ala.

9. The method, according to claim 8, wherein said analogue has a deletion of B30.

10. The method, according to claim 6, wherein at the 16th amino acid, the analogue is substituted by Ala.

11. The method, according to claim 10, wherein said analogue has a deletion at B30.

12. A pharmaceutical composition comprising an insulin analogue wherein the 16th or 26th amino acid of the B chain of human insulin (Tyr) is substituted by Ala, wherein said composition further comprises a pharmaceutical carrier.

13. The pharmaceutical composition, according to claim 12, wherein said analogue has a deletion at B1 (Phe) or a deletion at B30 (Thr), or a deletion at both B1 and B30.

14. The pharmaceutical composition, according to claim 12, wherein at the 26th amino acid, the analogue is substituted by Ala.

15. The pharmaceutical composition, according to claim 14, wherein said analogue has a deletion of B30.

16. The pharmaceutical composition, according to claim 12, wherein at the 16th amino acid, the analogue is substituted by Ala.

17. The pharmaceutical composition, according to claim 16, wherein said analogue has a deletion at B30.

Remarks

Claims 1 through 5 have been amended and new claims 6-17 have been added. No new matter has been added by these amendments.

The Commissioner is hereby authorized to charge any fees under 37 CFR 1.16 or 1.17 as required by this paper to Deposit Account 19-0065.

Respectfully Submitted



David R. Saliwanchik

Patent Attorney

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DRS/la

Marked-up Version of Amended Claims

Claim 1 (amended):

An insulin analogue wherein the 16th or 26th amino acid of the B chain of human insulin (Tyr) is substituted by Ala, and which optionally also comprises a deletion at B1(Phe) and/or B30 (Thr)[, for therapeutic use].

Claim 2 (amended):

The [An] insulin analogue, according to claim 1, wherein the 26th amino acid of the B chain of human insulin (Tyr) is substituted by Ala[(B26Ala)].

Claim 3 (amended):

The [An] insulin analogue, according to claim 1, wherein the 26th amino acid is substituted by Ala, and which comprises a deletion at B30[(des-B30, B26Ala)].

Claim 4 (amended):

The [An] insulin analogue, according to claim 1, wherein the 16th amino acid of the B chain of human insulin (Tyr) is substituted by Ala [(B16Ala)].

Claim 5 (amended):

The [An] insulin analogue, according to claim 1, wherein the 16th amino acid is substituted by Ala, and which comprises a deletion at B30 [(des-B30, B16Ala)].

Abstract of the Disclosure

Monomeric analogues of human insulin have a single substitution of the amino acid in 12th, 16th or the 26th position of the B chain of human insulin and may also have a terminal deletion in the B chain.

MONOMERIC ANALOGUES OF HUMAN INSULINField of the Invention

This invention relates to novel monomeric analogues of human insulin (HI) obtainable by recombinant DNA technology.

5 Background of the Invention

Insulin is highly effective in treating insulin-dependent diabetes, and has been used clinically for nearly 80 years. With advances in DNA technology and the development of biotechnology industries, insulin extracted from animal pancreas is gradually being replaced by recombinant forms of human insulin, produced in
10 microbial systems. This trend is encouraged by two observations; the number suffering from diabetes mellitus is on the increase globally and the clinical dose required to treat them is in milligram (mg) quantities.

Currently, the organisms employed for the commercial production of recombinant human insulin are *E. coli* and *S. cerevisiae*. The expression levels in *E.*
15 *coli* are high but difficulties associated with downstream purification often lead to loss of yield. These difficulties are not encountered with *S. cerevisiae*, because the insulin produced is secreted into the culture medium, facilitating purification. However, the level of expression observed in this organism is low and difficult to increase.

Until recently, introduction of Lispro®, clinical preparations of human insulin
20 contained polymeric forms of insulin which are slow-acting. Monomeric forms of insulin, as described in US-A-5618913, by contrast, are relatively fast-acting and mimic more closely the natural situation. They therefore demonstrate a great potential for clinical application. A commercial monomeric insulin, available as Lispro®, comprises inversion of amino acids 28 and 29 of the B chain of human insulin, and
25 may be abbreviated as B28Lys,B29Pro.

Kristensen *et al*, J. Biol. Chem. 272(20):12978-83 (1997), discloses alanine substitution at various positions on the insulin molecule, including B12, B16 and B26. A single substitution with Ala affected the binding activity of the resultant insulin analogue in certain cases.

30 Wang *et al*, Biochem. Mol. Biol. Int. 39(6):1245-54 (1996), discloses B12Thr, i.e. an insulin analogue in which the 12th amino acid of the B-chain of human insulin (Val) is substituted by Thr. Again, an effect on binding activity was observed.

EP-A-0046979 discloses des-B30 derivatives of human insulin.

EP-A-0291863 discloses des-B1 derivatives of human insulin.

Summary of the Invention

According to the present invention, novel human insulin analogues are monomeric variants of B12Thr, B16Ala and B26Ala; the latter have not previously been recognised as monomeric. In addition to replacement of any or all of the 12th, 16th and 26th amino acids on the B-chain, such that the analogue is monomeric, the B-1 and/or B-30 terminal amino acids may be absent. The term "insulin analogue" as used herein means a compound having a molecular structure similar to that of human insulin, including disulphide bridges between A7Cys and B7Cys and between A20Cys and B19Cys, and an internal disulphide bridge between A6Cys and A11Cys, and having insulin activity.

Without wishing to be bound by theory, it appears that, in the primary structure of the insulin molecule, a number of the amino acids in the B-chain are responsible for the polymerisation of insulin in clinical preparations. These include those in positions B12, B16 and B26. In particular, the replacement of Val by Thr in position B12 or Tyr by Ala in position B16 or B26 significantly reduces the tendency of the insulin analogues to polymerise even at high concentrations (see Example 9). This enhanced tendency to exist as a monomeric structure is not affected by deletion of either one or both of the terminal amino acids of the B-chain.

Description of the Invention

The Scheme, below, shows the construction of the expression plasmids pNHI-2/AOX1, pNHI-3/AOX1, pNHI-4/AOX1 and the engineering of recombinant cells YP99/NHI-2, YP99/NHI-3 and YP99/NHI-4. It sets out a representative procedure for the preparation of compounds of the invention, by analogy with the use of the human insulin target gene (HI) housed in the shuttle plasmid pHl/PGK. This shuttle vector is constructed from the plasmid pVT102-U (acquired from Canadian Research Institute) and subsequently multiplied by PCR (Maniatis *et al* (1989), Molecular Cloning A Laboratory Manual, 2nd ed. New York: Cold Spring Harbour Laboratory), to obtain multiple copies of human insulin target gene (HI) and flanking alpha mating factor leader (MFL) sequence. The target gene is then cloned into plasmid pPIC9 which is subsequently linearised with BglII prior to being employed to transform *P. pastoris* cell GS115 by the spheroplast method. Once plasmid pPIC9 containing the target gene is internalised, it integrates into the chromosomal DNA of the host cell [1]. Transformed cells bearing a high copy number of the HI gene are selected using the antibiotic G418 by the method described by Scover *et al* [2]. The presence of multiple copies of the HI are ascertained by the dot blotting method [3]. Cells bearing a high

copy number of the HI gene are utilised to generate the human insulin precursor by fermentation, and after purification converted to human insulin by tryptic transpeptidation.

In order to obtain recombinant forms of human insulin analogues according to this invention, target genes were produced. This was accomplished by the "gap double-stranded DNA" method described by Li Yiping *et al* [6] which permits site-directed mutations in the HI target gene. Primers specifically designed to give B12Thr, B16Ala and B26Ala were as follows;

For B12Thr (NHI-2): refer to Wang *et al*, *supra*

For B16Ala (NHI-3): 5' TGA GGC TTT GNN YTT GGT TTG CG 3' (SEQ ID No.1) in which N can be any nucleotide (G, A, T or C), and Y is C or G.

For B26Ala (NHI-4): 5' GAA AGA GGTT TTC NNY ACT CCT AGG GC 3' (SEQ ID No.2) in which N and Y are as defined above.

Novel human insulin analogues may be obtained by removing B30Thr and/or B1Phe, e.g. yielding a des-B1 and/or des-B30 analogue. Deletion may be achieved by known methodology. Rather than tryptic transpeptidation, to produce des-B30 human insulin, limited hydrolysis has been adopted, using trypsin in the preferred method, which further simplifies the process and increases the yield of insulin.

The methylotrophic yeast, *Pichia pastoris* is the preferred host for use in this invention for the preparation of insulin analogues because, as the Examples show, it has the advantages of high expression, simple processing, low production cost and high density culture. Furthermore it offers the advantages of a eukaryotic cell system; the correct folding and post-translational processing of secreted protein. These advantages greatly enhance the possibility of utilizing *P. pastoris* as the expression host in the scale-up of human insulin production. Its use in the expression of proteins of commercial importance has been documented elsewhere [3-5].

Human insulin analogues of the invention may be used in therapy. Their application and utility will be readily evident to those of ordinary skill in the art, e.g. in the treatment of diabetes mellitus.

Brief Description of the Drawings

Figure 1 shows the construction of pNHI-2/AOX1 plasmid of *Pichia pastoris*.

The following Examples illustrate the invention.

Example 1 Cloning of Mutated HI Gene

The plasmid pVT102-U from Canadian Biotechnology Research Institute was used to construct the plasmid pHI/PGK according to the standard method described

in Maniatis *et al* (1989). The construct pHl/PGK is a shuttle plasmid with phosphoglycerate kinase (PGK) promoter, followed by alpha mating factor leader sequence (MFL) to direct secretion of the product of the human insulin target gene (HI) flanked by a BamHI site at MFL 5' end and a HindIII site at HI 3' end. Using pHl/PGK as template, together with TCCGGATCCATGAGATTT (SEQ ID NO. 3) as the 5' primer and TGAATTCTTCTAGTTGCAGTAGTTT (SEQ ID NO. 4) as the 3' primer, DNA fragments containing MFL and HI with the BamHI site GGATCC at 5' end and the EcoR1 site GAATTC at the 3' end were obtained by PCR. To obtain DNA fragments containing MFL and the target gene NHI-2 (B12Thr), NHI-3 (B16Ala) and NHI-4 (B26Ala) the HI target gene in pHl/PGK plasmid was first mutated by site-directed mutagenesis then replicated by PCR. By inserting these fragments behind the AOX1 promoter of the plasmid pPIC9 (Invitrogen), expression plasmids pNHI-2/AOX1, pNHI-3/AOX1 and pNHI-4/AOX1 were obtained (see the Scheme and the accompany drawing; the latter shows the first plasmid, and the others may be prepared by the same procedure). The primers used to obtain the mutated genes in this invention have SEQ ID NOS. 1, 2 and 3.

Example 2 Construction and Screening of Expression Cell

The expression plasmids were linearised by BglII and used to transform *P. pastoris* cell GS115 (Invitrogen) using the spheroplast method. The linearised plasmids, once internalized, integrate into the chromosomal DNA of the host cell [1]. The recombinant cells, designated YP99/NHI-2, YP99/NHI-3 and YP99/HNI-4 with high copy number of the target gene, were selected by antibiotic G418 [2] and identified by the dot blotting method [3].

Example 3 Preparation of Precursors of HI analogues

High density fermentation was carried out in a 15 litre fermenter [7]. The following salt solutions were used in the fermentation: BSM - H_3PO_4 26.7 ml/l, $\text{CaSO}_4 \cdot \text{H}_2\text{O}$ 0.93 g/l, K_2SO_4 18.2 g/l, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 14.9 g/l, KOH 4.13 g/l; PTM1 - $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ 6 g/l, KI 0.08 g/l, $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ 3.0 g/l, $\text{NaMoO}_4 \cdot \text{H}_2\text{O}$ 0.2 g/l, H_3BO_3 0.02 g/l, $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ 0.5 g/l, ZnSO_4 20.0 g/l, H_2SO_4 5 ml/l, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 65.0 g/l.

Fermentation medium containing 6 L of salt solution BSM and 300 ml of glycerol is sterilised in the fermenter. Its pH is adjusted to 5.5 with 50% ammonium hydroxide. A 5 ml aliquot of salt solution PTM1 containing 1 mg of biotin is added per 1 litre of culture medium. The expression cell is inoculated to 50 ml YPG and grown in a shake flask at 30°C for 24 hr. The broth is added to 600 ml of YPG, shaken in 35 flasks for 24 hr, added to the culture medium and fermented for 24 hr to deplete

glycerol. Methanol solution containing PTM1 (5 ml/l) and biotin (1 mg/l) is added to induce the expression. The inductive fermentation is continued for 84 hr by feeding the above methanol solution. During the fermentation, the pH is maintained at 5.5 by adding 50% ammonium hydroxide. The expression level is measured by radioimmunoassay, SDS-polyacrylamide gel electrophoresis [8] and HPLC.

Example 4 Separation and Purification of the Precursors

The fermentation broth is centrifuged to remove the cell bodies. The supernatant is applied to a C8 column and purified by HPLC. After a single step of purification, a product can be obtained that is homogeneous in native polyacrylamide gel electrophoresis.

Example 5 Transpeptidation of the Precursors

Purified precursors of HI analogues from Example 4 are dissolved in DMSO/1,4-butanediol/H₂O (15:70:15, v/v) to a concentration of 30 mg/ml. Thr(Bu^t)-OBU^t is added in excess, and the pH is adjusted to 6.5 by ammonium hydroxide. TPCK-trypsin is added (substrate:enzyme = 5:1) and the reaction mixture is incubated at 25°C for 6 hr. The reaction is stopped by acidification. The product is precipitated using acetone, and purified by HPLC using C8 column.

Example 6 Preparation of des-B30 analogues

Purified precursors of HI analogues are dissolved in pH 8, 0.1M ammonium bicarbonate to a concentration of 10 mg/ml. TPCK-trypsin is added (substrate:enzyme = 200:1) and the reaction mixture is incubated at 25°C overnight. The product is analysed by native polyacrylamide gel electrophoresis

Example 7 Preparation of des-B1 analogues

HI analogues are reacted with phenylisocyanate in a molar ratio of 1:2, prior to treatment with trifluoroacetic acid as described by Bradenburg & Hoppe-Seyler, Physiol. Chem. 350:471. The products of this reaction are separated and analysed by electrophoresis and found to be almost exclusively des-B1 forms of insulin analogues.

Example 8 Preparation of des-B1, des-B30 analogues

Prepared by processing precursors of HI analogues as described in Example 6 followed sequentially by that described in Example 7.

Example 9 Determination of structural forms

The structural form of the recombinant human insulin analogues prior to deletion of the one or both terminal amino acids of the B-chain is determined electrophoretically. A preparation of each analogue is passed through Superdex G-75 column (HR 10/30). HI and [B28Lys, B29Pro] insulin (Lispro) are used as negative

and positive controls respectively. Phosphate buffered saline pH 7.4 is used as an elution buffer and the flow rate fixed at 0.4 ml/min. The concentration of the sample preparation is 1.2 mg/ml. The retention times and the peak profiles of human insulin analogues are shown in the following Table.

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Sample	Retention Time, min	Peak profile
HI	36.4	Unsymmetrical
[B28Lys, B29Pro]HI	39.4	Symmetrical
[B12Thr]HI	39.4	Symmetrical
[B16Ala]HI	38.3	Symmetrical
[B26Ala]HI	38.9	Symmetrical

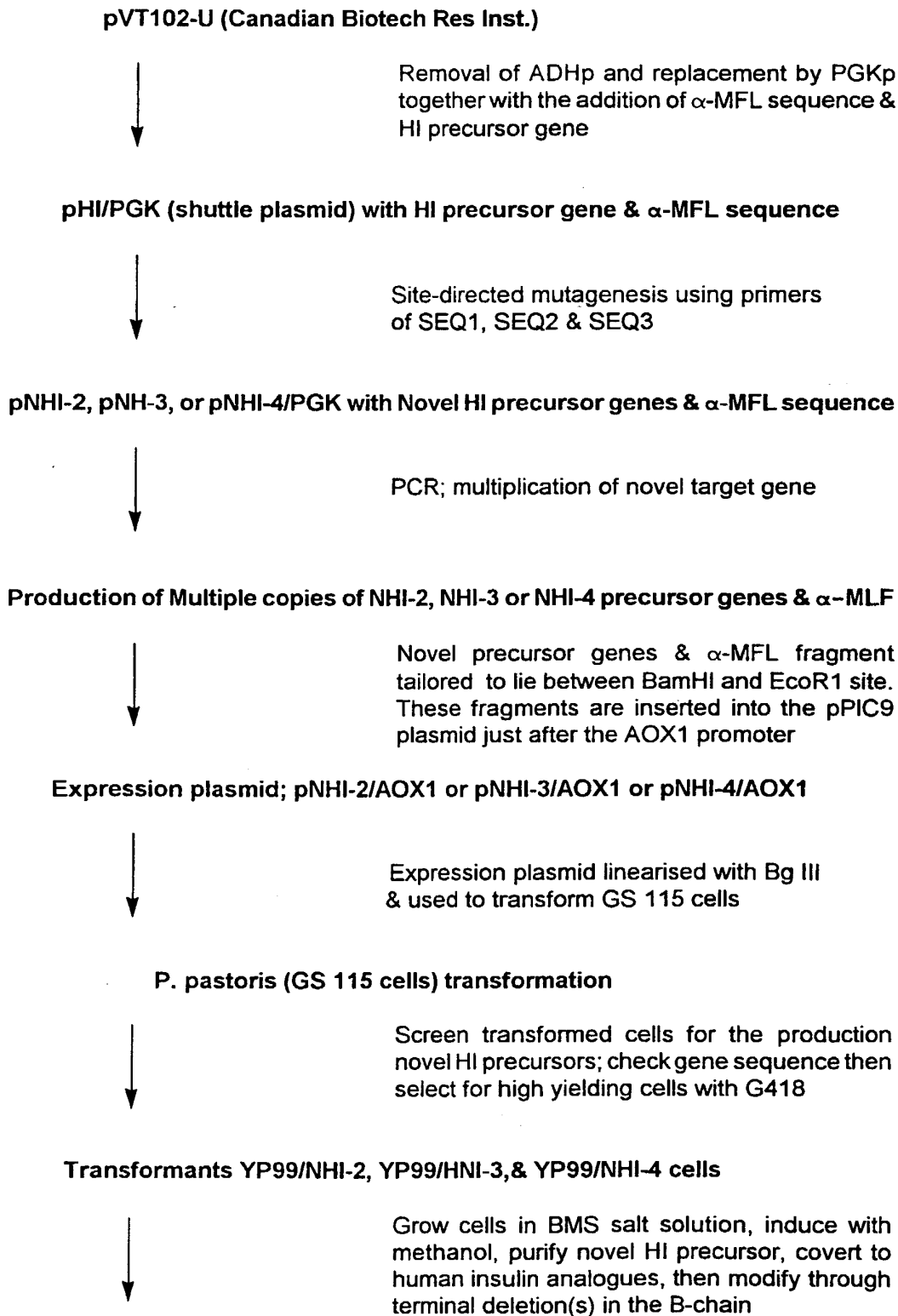
These results demonstrate that HI analogues B12Thr, B16Ala and B26Ala are all monomeric in form. They have a similar retention time and peak profile as the known positive control [B28Lys, B29Pro] human insulin.

15

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Scheme

CLAIMS

1. An insulin analogue wherein the 16th or 26th amino acid of the B chain of human insulin (Tyr) is substituted by Ala, and which optionally also comprises a deletion at B1(Phe) and/or B30 (Thr), for therapeutic use.
- 5 2. An insulin analogue wherein the 26th amino acid of the B chain of human insulin (Tyr) is substituted by Ala (B26Ala).
3. An insulin analogue according to claim 1, wherein the 26th amino acid is substituted by Ala, and which comprises a deletion at B30 (des-B30, B26Ala).
4. An insulin analogue wherein the 16th amino acid of the B chain of human
10 insulin (Tyr) is substituted by Ala (B16Ala).
5. An insulin analogue according to claim 1, wherein the 16th amino acid is substituted by Ala, and which comprises a deletion at B30 (des-B30, B16Ala).

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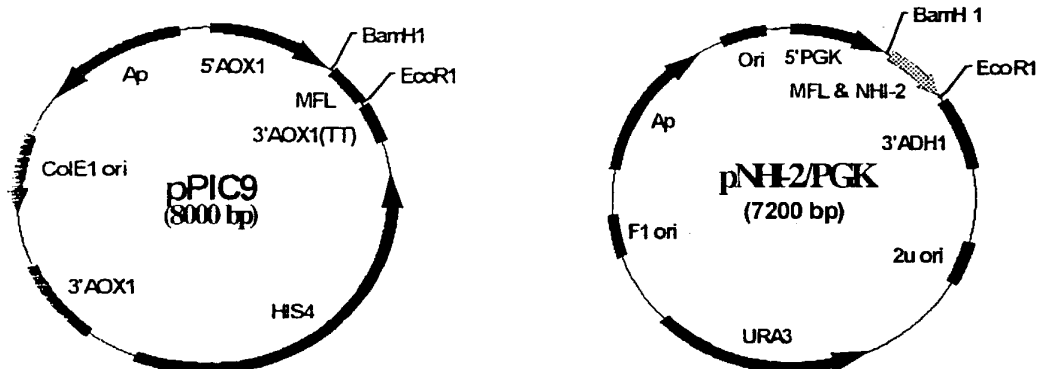
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(54) Title: MONOMERIC ANALOGUES OF HUMAN INSULIN

(57) Abstract: Monomeric analogues of human insulin have a single substitution of the amino acid in 12th, 16th or the 26th position of the B chain of human insulin and may also have a terminal deletion in the B chain.

1/1



Bam HI, EcoR1

Bam HI, EcoR1

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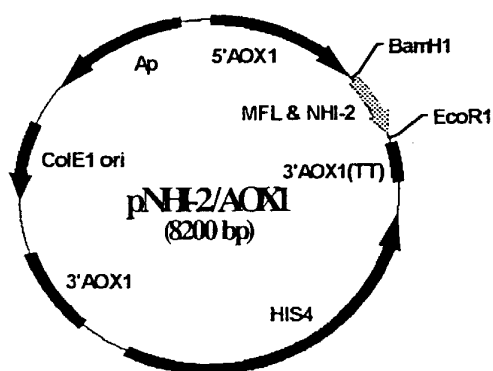


Figure 1

USA

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MONOMERIC ANALOGUES OF HUMAN INSULIN

the specification of which ☐ is attached hereto or

☒ was filed on 08 SEP 2000 as United States Application Number or PCT International Application Number PCT/GB00/03460 and was amended on 19 NOV 2001 (if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above. I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR 1.56. I hereby claim foreign priority benefits under 35 U.S.C. 119(a)-(d) or 365(b) of any foreign application(s) for patent or inventor's certificate, or 365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for a patent or inventor's certificate, or PCT international application having a filing date before that of the application on which priority is claimed:

Prior Foreign Application Number(s)	Country	Foreign Filing Date	Priority Not Claimed	Certified Copy Attached?	
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